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ON THE OCCURRENCE OF THERMOSTABLE AND SIMPLE BACTERICIDAL AND OPSONIC SUBSTANCES.*

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IN an article entitled "Further Studies on Virulent Pseudo-diphtheria Bacilli" published two years ago,[1] Dr. Horton and I described the properties of the serum of animals immunized against the "Ruediger bacillus," an organism belonging probably to the pseudo-diphtheria group. The serum of rabbits and of goats which had been immunized against the bacillus presented certain peculiarities. It contained specific agglutinin, opsonin, and bacteriolysin for these bacilli and showed itself extraordinarily resistant to heat, age, light, and drying. After the serum had been partially or wholly inactivated by heat, it was found to be impossible to reactivate it by adding fresh complement. Experiments in removing amboceptor by absorption also failed, and the bactericidal substance apparently did not consist of complement and amboceptor. The opsonin was also found to be highly resistant to heat and attempts to reactivate heated serum failed, but opsonin and bacteriolysin were apparently not the same substance.

The conclusions drawn at the end of the work were as follows: "The study of the bacteriolysin and opsonin of our immune serum reveals the fact that we have here an immune serum of high degree of thermostability, apparently not containing complement and amboceptor.

"Experiments on guinea-pigs indicate that the immune serum causes a marked polynucleosis and increases phagocytosis *in vivo* as well as *in vitro*, hence the protective action of this serum must be ascribed in part at least to the immune opsonin.

"The bacteriolysin and opsonin of these immune sera are not the same substance."

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* Received for publication October 31, 1908.

[1] *Jour. Infect. Dis.*, 1906, 3, p. 128.

The advances made in the last two years in our knowledge of antibodies, especially the opsonins, made it seem advisable to review and extend the work on this serum with regard to the two disputable points; namely, that the bacteriolysin and opsonin do not contain complement or amboceptor, and second, that the thermostable bacteriolysin is not identical with the thermostable opsonin.

There are a number of recent publications dealing with heat-resistant antibodies, hemolytic and bactericidal, some being even "coctostable," resisting the temperature of boiling water. Thus Korschun and Morgenroth,[1] Woelfel,[2] Levaditi,[3] and Conradi[4] have all described heat-resistant lytic substances extracted from animal cells. These bodies are not complex, i. e., not resolvable into an amboceptor-complement group. Other thermostable antibodies, both hemolysins and bacteriolysins, have been shown to consist of complement and amboceptor. Such are the endolysins of Schattenfroh[5] and Pettersson,[6] thermostable bodies extracted from disintegrated leucocytes. A bactericidal blood serum that owes this property to a substance which is thermostable and not complex has not been described so far, and it is with some diffidence that I claim these properties for the serum studied in this paper.

NATURE OF THE BACTERICIDAL SUBSTANCE.

The serum of rabbits is normally bactericidal for the "Ruediger bacillus," although not strongly so. Plates made from tubes containing one part of normal serum to four of broth usually show a diminution of one-half to one-fourth of the number of colonies, when compared with the plates from control tubes. The bacteriolysin increases greatly after immunization, reaching in some instances 80 times its original strength. This increase is not, however, steady and continuous, but occurs in waves, the low-water mark falling to normal, or lower, and this even after repeated injections. Typically there is a rise at each injection followed by a fall and occasionally preceded by one, i. e., a negative phase. The curve in Chart 1 shows the variations in the bactericidal substance from day to day, and serves

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[1] *Berl. klin. Wchnschr.*, 1902, 39, p. 870.

[2] *Jour. Infect. Dis.*, 1905, 2, p. 97.

[3] *Ann. de l'Inst. Pasteur*, 1903, 17, p. 187.

[4] *Beitr. z. chem. Physiol. u. Pathol.*, 1901, 1, p. 516.

[5] *Münch. med. Wchnschr.*, 1898, 45, p. 1109.

[6] *Centralbl. f. Bakt.*, 1907, 45, p. 237.

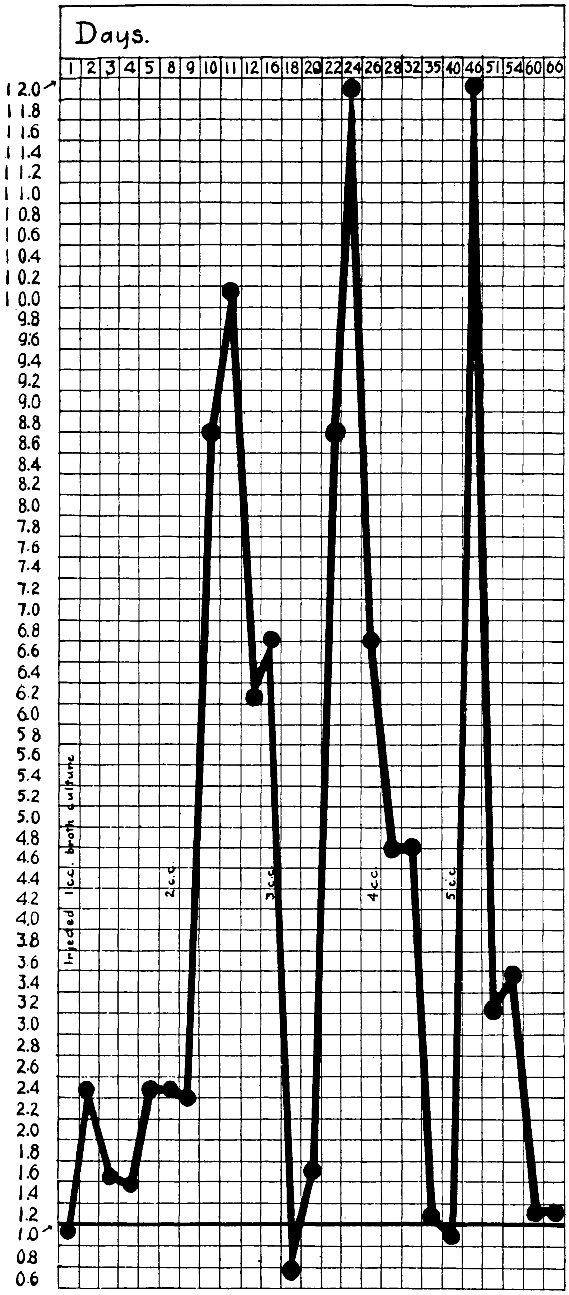

CHART 1.—Curve showing fluctuations of bactericidal substance in the blood of a rabbit during immunization.

to explain why, in our former work, we sometimes obtained sera of very low bactericidal power from animals supposedly highly immune.

The amount of bacteriolysin present was estimated by comparing the plates made from tubes containing immune serum with the plates. made from similar tubes containing serum from two normal rabbits. Thus, if these control plates contained 500 colonies, and the plates from the tubes with immune serum contained only 50 colonies, the bactericidal power of the immune serum would be 10.

Goat serum differs from rabbit serum in that it is normally strongly bactericidal to this bacillus, and immunization has practically no effect in increasing the amount of bacteriolysin. Three goats were injected with the same quantities of broth culture of the Ruediger bacillus, and in all the result was the same, a very slow and insignificant increase in the bactericidal substance.

The bactericidal substance is stable, not only in immune serum, but also in normal serum. Normal rabbit serum loses its bactericidal power only after one week's exposure to daylight, at room temperature, and normal goat serum can endure 12 days. Both can be heated to 70° for 10 minutes without complete inactivation. The immune serum of both animals is still more resistant to heat and age. Immune rabbit serum is not inactivated by exposure to direct daylight for a period shorter than 15 days. When kept in the ice-box, immune goat serum has retained its bactericidal power for more than four months, provided it is left in contact with the clot, deterioration taking place more rapidly if the serum is removed from the clot. Immune rabbit serum dried to powder and redissolved in broth is highly bactericidal. As to the effect of heat, immune serum resists long exposure to high temperature, the difference between normal and immune serum being shown not so much in the degree of heat which can be borne but in the period of time which the exposure can be prolonged without complete inactivation. Thus normal serum can be heated to 70° C. for 10 minutes without complete inactivation, and immune serum is inactivated by exposure for the same length of time to 80°, but normal serum is destroyed by two hours' exposure to 55°, while immune rabbit serum can survive 24 hours at this temperature and immune goat serum, three days. The increase in the bactericidal substance of the goat serum after immunization is thus made plainly evident by applying the heat test. For, while the unheated serum may show no increase in bactericidal power over unheated normal serum, yet the latter will be completely inactivated by two hours at 55° while the former will have lost very little of its activity. The loss in bacteriolysin by heat is progressive, and the difference in heat resistance between goat and rabbit serum—the serum of the goat retains its power longer than that of the rabbit—is probably only a quantitative one.

It will be seen, then, that we have here a normal lysin of a high degree of thermostability, and that this lysin or lytic property of the serum increases under immunization. According to Dean, this should be explained by the fact that while the normal serum contains both complement and amboceptor the latter is present in small quantities only, and therefore normal serum is quickly inactivated. In immune serum, on the other hand, amboceptor is present in large quantities and heating, which destroys complement, causes only partial inactivation, because the amboceptor is capable of acting alone. The high degree of thermostability of our normal serum, however, argues against the presence of complement, and the experiments made in reactivation confirm the belief that the bactericidal substance in both immune and normal serum is simple and indivisible.

Various methods were used in attempts to determine the presence of complement and amboceptor. Exposure of a mixture of serum and bacilli to zero temperature resulted in the absorption of the bactericidal substance *in toto*. Bacilli which had been treated with immune serum at zero were removed, washed, and incubated, some with normal salt solution only, some with the addition of fresh normal rabbit serum. There was no difference between the two sets of plates except what could be accounted for by the bactericidal action of the normal serum.

In attempting to reactivate heated normal serum, the serum of sheep and human beings was chosen, as both are highly bactericidal to the Ruediger bacillus, and the serum of immune goats and rabbits was used for testing immune serum. The serum was heated to 64°–70° for 10 to 30 minutes. As complement, the substances employed by different observers were used. Fresh serum in the proportions used by Dean, fresh living leucocytes, according to the method of Schneider,[1] and disintegrated leucocytes, according to Pettersson's method, were added to the inactivated serum. The following is the result obtained in a typical experiment with inactivated immune serum and fresh complementary serum.

TABLE 1.

Mixtures		Colonies after 4 Hrs. at 37° C
Broth	1.0	6,400
Normal rabbit serum	0.2+Broth 0.8	2,450
Immune goat serum	" + " "	2,000
Rabbit serum 1 part Immune goat serum 70° 2 parts	" + " "	2,240
Rabbit serum 2 parts Immune goat serum 70° 2 parts	" + " "	1,672
Rabbit serum 2 parts Immune goat serum 70° 1 part	" + " "	1,404
Rabbit serum 4 parts Immune goat serum 70° 1 part	" + " "	1,100
Unheated Immune goat serum	" + " "	600

There is evidently no real reactivation here. The next to the last tube in the series contained the proportions of serum recommended by Dean, namely, one part of inactivated to four parts of fresh serum, and here the number of colonies fell to one-half of the average for the two sera used separately. This result was invariably obtained in these experiments, a greater bacteriolytic effect from the use of the two sera in these proportions than from any other combination. Nevertheless, this can hardly be accounted reactivation, for the two sera are present in proportions exactly the reverse of those usual in reactivation experiments, and the resulting combination is only half as strong as the original unheated serum. Probably we have here a summation of effects due to the combination of two sera, both of which have some bacteriolytic power.

Schneider has described, under the name of "Leukine," certain bactericidal substances, which are secreted by living leucocytes under the stimulus afforded by inactivated immune serum. His experiments were repeated with leucocytes from the dog, rabbit, and guinea-pig, and with immune serum of goat and rabbit inactivated by heat. In each instance the phagocytic power of the leucocytes was tested in order to make sure that they were uninjured and active, but the result of these experiments was always negative; it was not possible to reactivate the serum by this method.

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[1] *Münch. med. Wchnschr.*, 1908, 55, p. 499.

According to Pettersson there are three substances involved in bactericidal immunity, namely, opsonin, serum bacteriolysin, which is thermolabile and complex, and finally endolysin, a complex but thermostable body contained in the bodies of leucocytes, and recoverable from these after they have been subjected to repeated freezing and thawing. In order to discover whether the thermostable bacteriolysin of our serum were derived from the leucocytes, blood was drawn from a normal sheep, from an immune goat, and from an immune rabbit. The blood was defibrinated by beating, the serum removed immediately, and the upper layer of corpuscles withdrawn and freed from serum by washing in normal salt solution. A portion of these corpuscles was heated to 68° C., and another portion was frozen and thawed three times; part of this latter was then heated to 68°. The bactericidal effect of serum, corpuscles, and frozen corpuscles was compared both before and after heating to 68°. An attempt also was made to reactivate the heated serum by means of frozen leucocytes, and the effect of unheated serum plus frozen leucocytes was compared with the effect of unheated serum plus uninjured leucocytes. Without giving the details of these experiments it will be sufficient to say (*a*) that the extract from frozen leucocytes was usually more strongly bactericidal than the suspension of uninjured leucocytes, but never so strong as the serum; (*b*) that the leucocytic extract was not more heat resistant than the serum, but less so, probably a purely quantitative difference; (*c*) that mixtures of heated serum and frozen leucocytes showed merely a summation of effects, no reactivation, and that (*d*) the addition of leucocytes, either living or disintegrated, to the unheated serum produced a mixture more strongly bactericidal than the serum alone. This last fact is in accordance with the observation repeatedly made in the course of this work, that serum removed from the clot by defibrination was less strongly bactericidal than serum left in contact with the clot. Undoubtedly the bactericidal substance is in part derived from the bodies of leucocytes.

As a result of these experiments it seems justifiable to assert that the substance in normal and immune serum bactericidal to the Ruediger bacillus is non-complex, and cannot be resolved into complement and amboceptor.

The bactericidal substance was precipitated from human serum and from immune rabbit serum by the addition of twenty parts of 98 per cent alcohol. The precipitate and the filtrate were both evaporated to dryness in the incubator, were then redissolved in broth, and tested:

TABLE 2.

Mixtures		Colonies after 4 Hrs. at 73°
Control broth	1.0	2,200
Filtrate in broth	1.0	2,500
Precipitate in broth	1.0	640

NATURE OF THE OPSONIN.

The normal serum of the rabbit and of the goat contains opsonin for the Ruediger bacillus, goat serum averaging an index of about 1.5 as compared with rabbit serum. Normal opsonin deteriorates quickly, disappearing after three or four days' exposure to room temperature. It practically is destroyed by 15 minutes' exposure to 70°, by 30 minutes' exposure to 60°, and two hours' exposure to 45° C. In goat serum the degree of resistance is a little higher than in rabbit serum, probably a quantitative difference. On immunization the amount of opsonin increases in the typical manner, describing the sort of curve which we expect to follow injections of immunizing doses. Goat serum does not respond as well as rabbit serum in the formation of opsonin. Chart 2 shows the opsonic index of a rabbit immunized against the Ruediger bacillus. Immune opsonin is resistant to heat and to drying, although not to the same degree as immune bacteriolysin. The latter is still active after four hours' exposure to 64°, but immune opsonin is destroyed by two hours' exposure to that temperature.

This is a point of difference, however, upon which one would hesitate to lay stress, for it is well known that the Wright method of determining opsonin does not show small quantities of the substance while even a small amount of bacteriolysin is easily demonstrable.

Dean and others, notably Chapin and Cowie,[1] have succeeded in restoring the opsonic power to heated serum by the addition of fresh normal serum. Experiments in restoring the opsonin to our inactivated serum were as unsuccessful as the experiments in restoring bacteriolysin. The following is a typical experiment with heated immune goat serum and fresh normal rabbit serum:

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[1] *Jour. Med. Res.*, 1907, 17, pp. 37, 95, 213.

TABLE 3.

Mixtures	Average No. of Bacilli per Leucocyte	
Rabbit serum	2.2	2.36
Goat serum heated to 70°	0.16	
1 part rabbit serum + 1 part goat serum 70°	3.75	
1 " " " 2 " " " "	2.25	
1 " " " 3 " " " "	2.8	
1 " " " 4 " " " "	1.3	
2 " " " 1 " " " "	3.75	
3 " " " 1 " " " "	2.8	
4 " " " 1 " " " "	4.3	

Table 3 shows that phagocytosis is greatest in the tube containing the largest quantity of unheated serum, and least in that containing the smallest quantity. In the last tube we have almost twice as much phagocytosis as in the control, and this is in the tube containing the proportions recommended by Dean, namely, one part of amboceptor to four of complement; but here, as in the case of bactericidal experiments, one would hesitate to attribute the effect to reactivation.

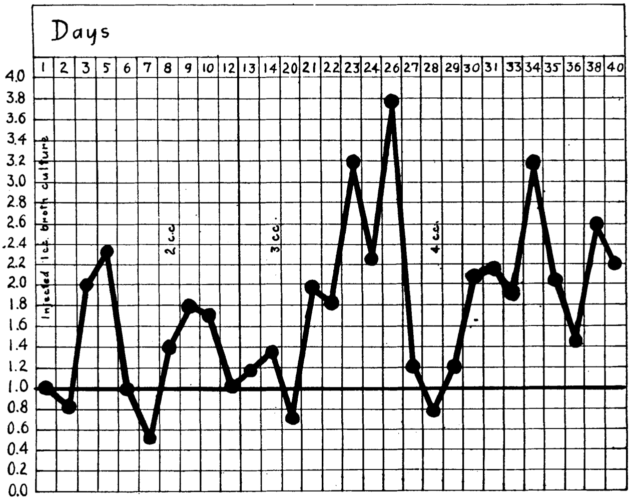

CHART 2.—Opsonic index of rabbit during immunization.

Like the bacteriolysin, the opsonin is precipitated by the addition of 98 per cent alcohol.

TABLE 4.

Mixtures	Average per Leucocyte
Serum	5.8
Filtrate dried and dissolved in normal salt solution	0.4
Precipitate " " " " " "	4.0

The question as to whether the bactericidal and the opsonic properties of a given serum are due to the action of one body or of two distinct bodies is still unsettled. Dean maintains that they depend on one and the same substance, on the ground that sera which are strongly bacteriolytic for a certain organism are also strongly osponic for that organism, both producing their effect through the combined action of a thermolabile complement and a thermostable amboceptor. The latter, Dean finds, is capable of acting alone after the complement has been destroyed by heat, but its effect is strengthened by the addition of fresh complement. This is true of both opsonin and bacteriolysin.

On the other hand, Neufeld and Hüne[1] argue against the identity of the two substances on the ground of a study of typhoid and paratyphoid sera. The serum of animals immunized against paratyphoid bacilli is not bactericidâl to those organisms, but does contain specific opsonin for them. The serum of a typhoid patient may be bactericidal to typhoid bacilli and contain no opsonin at all, or but very little, while another, no more strongly bactericidal, may contain a decided amount of opsonin. Therefore, they conclude, in these two instances the opsonin and bacteriolysin are not identical.

Hektoen[2] in 1906 reviewed the literature previous to that date and, adding the result of his own researches, concluded that opsonins are distinct from bactericidal substances and from agglutinins for various reasons but more particularly because serum, normal as well as immune, may contain opsonin for a given organism, but not, at least as far as is yet known, the proper lytic amboceptor for that organism.

Pettersson also maintains the duality of these substances as does Schneider.

In the case of our immune serum there are several arguments in favor of the identity of the bactericidal substance and the opsonin. In the first place, both are heat resistant, both show an intermittent increase during immunization, both are apparently non-complex bodies, incapable of reactivation, both are held back by a fine filter and precipitated by alcohol. On the other hand, there are cogent arguments against their identity.

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[1] *Arb. a. d. kaiserl. Gesund.*, 1907, 25, p. 164.

[2] *Jour. Infect. Dis.*, 1906, 3, p. 434.

If the two substances were identical, then any serum which was rich or poor in one should be rich or poor in the other also, but this is not found to be the case. Human serum has an opsonic index only slightly above rabbit serum, but is far more strongly bactericidal, sometimes a hundred times more. Moreover, two specimens of human serum which have approximately the same opsonic index may differ in bactericidal power as much as 12 to 200.

The following table gives the opsonic and bactericidal indices of two human beings and of seven lower animals, the serum of rabbits being used as standard.

TABLE 5.

Serum	Bactericidal Index	Opsonic Index
Human No. 1	12.	1.3
Human No. 2	200.	1.2
Chicken	50.	1.2
Goat	20.	1.5
Rat	16.	5.0
Horse	12.	0.67
Sheep	7–10	0.5
Guinea-pig	2.5–3	1.0
Dog	2.0	0.3

The bactericidal content of the sera varies more than the opsonic, but it is plain that the two do not keep pace with each other, that a high opsonic index does not mean a high bactericidal index.

Again, though the bactericidal substance undergoes an intermittent, wavelike increase on immunization, as does the opsonin, yet the two curves do not correspond, but a low opsonic index may be coincident with a high bactericidal index and *vice versa.* Chart 3 shows the three curves, opsonic, bacteriolytic, and agglutinative, of the serum of a goat, and Chart 4 the same of the serum of a rabbit. These indicate that while the three antibodies are formed simultaneously they are not identical, for they do not vary at the same rate. The difference is shown even more strikingly when one studies the processes which occur in guinea-pigs injected with a lethal dose of the Ruediger bacillus and with a protective dose of immune serum.

Dr. Horton and I found that phagocytosis played a more or less important rôle in the protection of guinea-pigs against injections of this organism and that an increase of polymorphonuclear leucocytes occurs very quickly after an injection and is much greater in the guinea-pig which has received a protective dose of serum than in one which is not so protected. Two guinea-pigs were injected with lethal doses and at the end of three hours these two and a control animal were bled and a differential count of leucocytes made, with this result:

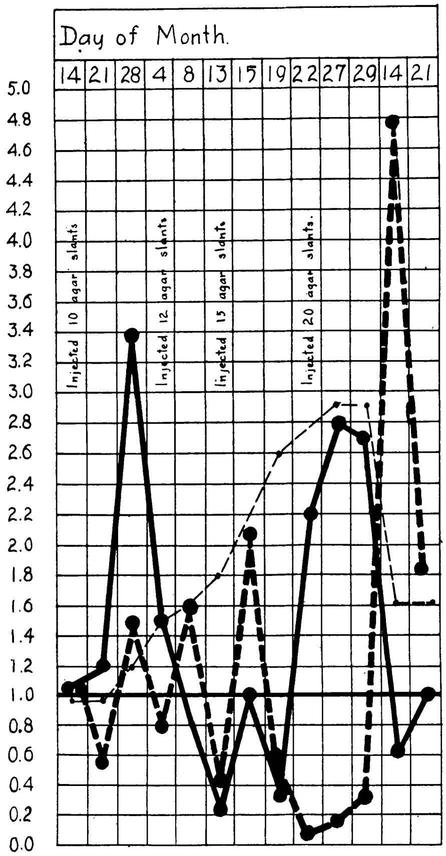

CHART 3.—Comparison of opsonin (solid line), bacteriolysin (heavy broken line), and agglutinin (fine broken line) in the blood of a goat during immunization.

TABLE 6.

	Per cent Polynuclears
Control guinea-pig	1.6
Guinea-pig injected with broth culture Ruediger bacillus	8.6
Guinea-pig injected with broth culture Ruediger bacillus plus immune serum	44.0

It was also found that the peritoneal exudate of the protected guinea-pig contained a far larger number of polymorphonuclear leucocytes than did that of the unprotected animal. Guinea-pig A was killed three hours after receiving intraperitoneally 4 c.c. of a broth culture of B. No. 4. The exudate was almost clear with many bacilli and but few leucocytes, 2 per cent of which were polynuclears, 94 per cent small mononuclears, and 4 per cent large mononuclears. Guinea-pig B received in addition to 4 c.c. of broth culture of B. No. 4, 2 c.c. of immune serum. It was killed at the end of three hours. The peritoneal exudate was slightly cloudy and contained a few agglutinated bacilli and many leucocytes, 54 per cent of which were polynuclears, 29 per cent small, and 12 per cent large mononuclears.

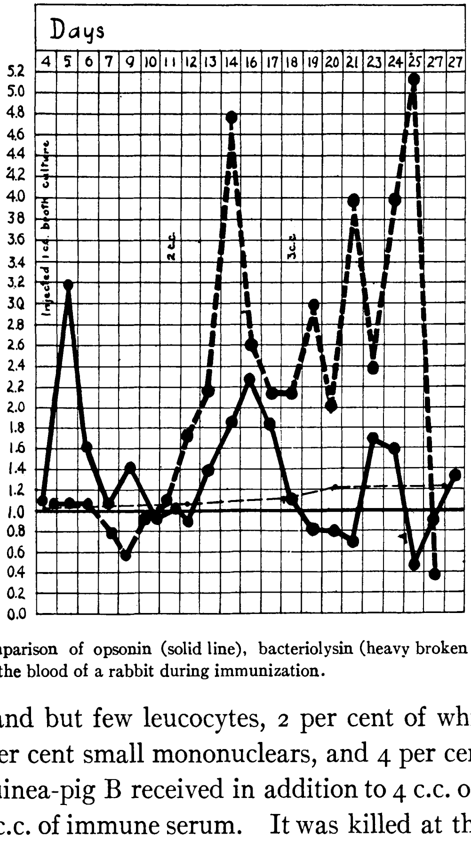

CHART 4.—Comparison of opsonin (solid line), bacteriolysin (heavy broken line), and agglutinin (fine broken line) in the blood of a rabbit during immunization.

This increase in number of the polymorphonuclear leucocytes occurs, early after the injection of culture plus protective serum. The withdrawal of small amounts of fluid from the peritoneal cavity of protected guinea pigs at short intervals after injection showed that phagocytosis of bacilli begins between one-half and one hour after the injection, reaches its height at the end of two and one-half hours, and is ended after six hours. The destruction of the bacilli is, however, much slower. Guinea-pigs which had been given a lethal dose of culture and a protective dose of immune rabbit serum were killed at different periods after the time of injection and cultures made from the blood and the organs. At the end of three hours, at the time when phagocytosis is at its height, a general invasion of bacilli has occurred and all body fluids and organs yield positive cultures. At the end of six hours cultures are obtained from the spleen and peritoneal cavity only, and at the end of 18 hours all cultures are sterile. Apparently then the opsonin is the protective substance first formed, the bactericidal substance later. Chart 5, which gives the curves of these antibodies in the blood of a guinea-pig after one injection of culture and immune serum, bears out this conclusion. The opsonin had increased at the end of three hours, during which time there was no increase of bacteriolysin, rather a slight fall. At the end of 20 hours the bacteriolysin was at its height and from then on it declined, while the opsonin, which was low at the end of 20 hours, rose again on the following day, as the bacteriolysin was falling.

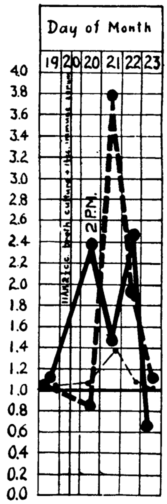

CHART 5.—Comparison of opsonin (solid line), bacteriolysin (heavy broken line), and agglutinin fine broken line) in the blood of a guinea-pig following the injection of 2.5 c.c. of broth culture of the Ruediger bacillus and 1.5 c.c. of immune rabbit serum.

It is possible that this bacteriolysin is of the nature of a toxin in Ehrlich's sense. In that case, it might be possible to produce an antitoxin by injections of immune serum. This has been attempted by injecting rabbits with immune goat and immune rabbit serum and guinea-pigs with immune goat serum, but the attempt thus far has proved a failure and there has been no production of antibacteriolysin.

SUMMARY.

In the blood of human beings and of some of the lower animals there is a thermostable bactericidal substance and thermostable opsonin for the Ruediger bacillus. These substances are increased on immunization and then show a much higher degree of thermostability.

The bacteriolysin of normal and of immune serum suffers a gradual loss on heating, but there is nothing to indicate that this is due to the loss of complement.

Experiments in reactivation of heated serum by means of fresh serum, of living leucocytes, and of disintegrated leucocytes show that the bacteriolysin and opsonin are not composed of complement and amboceptor.

The opsonin and bacteriolysin have many other properties in common, such as thermostability and precipitability by alcohol, but they are shown to be distinct by the fact that a serum rich in one of them may be poor in the other, and the further fact that while immunization causes an intermittent increase of both opsonin and bacteriolysin, the curves described by the two bodies are not parallel.

Experiments on guinea-pigs show that the first result of the injection of a broth culture of the Ruediger bacillus and a protective dose of immune serum is an increase of polymorphonuclear leucocytes in the circulating blood, the appearance of large numbers of phagocytes in the peritoneal cavity, and a rise in the opsonic index. At this time there is no demonstrable increase of bacteriolysin. Later on, the latter begins to increase and reaches its height after the opsonin has declined and phagocytosis has ceased. A second rise in the opsonic curve then occurs without any corresponding rise in the bacteriolysin.

It has not been possible to produce an antibacteriolytic serum by injections of immune serum in rabbits and guinea-pigs.